

SPECIFICATION

Title of the Invention

Process for Producing Microbial Transglutaminase

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Background of the Invention

The present invention relates to a protein having a transglutaminase activity, DNA which encodes for the protein, and a process for producing the protein. In particularly, the present invention relates to a process for producing a protein having a transglutaminase activity by a genetic engineering technique.

Transglutaminase is an enzyme which catalyzes the acyl transfer reaction of a γ -carboxyamido group in a peptide chain of a protein. When such an enzyme react with the protein, a reaction of an ϵ -(γ -Glu)-Lys forming reaction or substitution reaction of Gln with Glu by the deamidation of Glu can occur.

The transglutaminase is used for the production of gelled foods such as jellies, yogurts, cheeses, gelled cosmetics, etc. and also for improving the quality of meats [see Japanese Patent Publication for Opposition Purpose (hereinafter referred to as "J. P. KOKOKU") No. Hei 1-50382]. The transglutaminase is also used for the production of a material for microcapsules having a high thermal stability and a carrier for an immobilized enzyme. The transglutaminase is thus industrially very useful.

As for transglutaminases, those derived from animals and those derived from microorganisms (microbial transglutaminase; hereinafter

referred to as "MTG") have been known hitherto.

5 The transglutaminases derived from animals are calcium ion-
dependent enzymes which are distributed in organs, skins and bloods of
animals. They are, for example, guinea pig liver transglutaminase
[K.Ikura et al., Biochemistry 27, 2898 (1988)], human epidermis keratin
cell transglutaminase [M. A. Philips et al., Proc. Natl. Acad. Sci. USA
87, 9333 (1990)] and human blood coagulation factor XIII (A. Ichinose
et al., Biochemistry 25, 6900 (1990)).

10 As for the transglutaminases derived from microorganisms, those
independent on calcium were obtained from microorganisms of the genus
Streptovorticillium. They are, for example, Streptovorticillium
griseocarneum IFO 12776, Streptovorticillium cinnamoneum sub sp.
cinnamoneum IFO 12852 and Streptovorticillium mobaraense IFO 13819 [see
15 Japanese Patent Unexamined Published Application (hereinafter referred
to as "J. P. KOKAI") No. Sho 64-27471].

20 According to the peptide mapping and the results of the analysis
of the gene structure, it was found that the primary structure of the
transglutaminase produced by the microorganism is not homology with
that derived from the animals at all (European Patent publication No. 0
481 504 A1).

25 Since the transglutaminases (MTG) derived from microorganisms
are produced by the culture of the above-described microorganisms
followed by the purification, they had problems in the supply amount,
efficiency, and the like. It is also tried to produce them by a
genetic engineering technique. This technique includes a process which
is conducted by the secretion expression of a microorganism such as E.

coli, yeast or the like (J. P. KOKAI No. Hei 5-199883), and a process wherein MTG is expressed as an inactive fusion protein inclusion body in E. coli, this inclusion body is solubilized with a protein denaturant, the denaturant is removed and then MTG is reactivated to obtain the active MTG (J. P. KOKAI No. Hei 6-30771).

However, these processes have problems when they are practiced on an industrial scale. Namely, when the secretion by the microorganisms such as E. coli and yeast is employed, the amount of the product is very small; and when MTG is obtained in the form of the inactive fusion protein inclusion body in E. coli, an expensive enzyme is necessitated for the cleavage.

It is known that when a foreign protein is secreted by the genetic engineering method, the amount thereof thus obtained is usually small. On the contrary, it is also known that when the foreign protein is produced in the cell of E. coli, the product is in the form of inert protein inclusion body in many cases although the expressed amount is high. The protein inclusion body must be solubilized with a denaturant, the denaturing agent must be removed and then MTG must be reactivated.

It is already known that in the expression in E. coli, an N-terminal methionine residue in natural protein obtained after the translation of gene is efficiently cleaved with methionine aminopeptidase. However, the N-terminal methionine residue is not always cleaved in an exogenous protein.

Processes proposed hitherto for obtaining a protein free from N-terminal methionine residue include a chemical process wherein a protein

having methionine residue at the N-terminal or a fusion protein having a peptide added thereto through methionine residue is produced and then the product is specifically decomposed at the position of methionine residue with cyanogen bromide; and an enzymatic process wherein a recognition sequence of a certain site-specific proteolytic enzyme is inserted between a suitable peptide and an intended peptide to obtain a fusion peptide, and the site-specific hydrolysis is conducted with the enzyme.

However, the former process cannot be employed when the protein sequence contains a methionine residue, and the intended protein might be denatured in the course of the reaction. The latter process cannot be employed when a sequence which is easily broken down is contained in the protein sequence because the yield of the intended protein is reduced. In addition, the use of such a proteolytic enzyme is unsuitable for the production of protein on an industrial scale from the viewpoint of the cost.

Conventional processes for producing MTG have many problems such as supply amount and cost. Namely, in the secretion expression by *E. coli*, yeast or the like, the expressed amount is disadvantageously very small. In the production of the fusion protein inclusion body in *E. coli*, it is necessary, for obtaining mature MTG, to cleave the fusion part with restriction protease after the expression. Further, it has been found that since MTG is independent on calcium, the expression of active MTG in the cell of a microorganism is fatal because this enzyme acts on the endoprotein.

Thus, for the utilization of MTG, produced by the gene

recombination, on an industrial scale, it is demanded to increase the production of mature MTG free of the fusion part. The present invention has been completed for this purpose. The object of the present invention is to product MTG in a large amount in microorganisms such as E. coli.

When MTG is expressed with recombinant DNA of the present invention, methionine residue is added to the N-terminal of MTG. However, by the addition of the methionine residue to the N-terminal of MTG, there is some possibility wherein problems of the safety such as impartation of antigenicity to MTG occur. It is another problem to be solved by the present invention to produce MTG free of methionine residue corresponding to the initiation codon.

Summary of the Invention

An object of the present invention is to provide a novel protein having a transglutaminase activity.

Another object of the present invention is to provide a DNA encoding for the novel protein having a transglutaminase activity.

Another object of the present invention is to provide a recombinant DNA encoding for the novel protein having a transglutaminase activity.

Another object of the present invention is to provide a transformant obtained by the transformation with the recombinant DNA.

Another object of the present invention is to provide a process for producing a protein having a transglutaminase activity.

These and other objects of the present invention will be

apparent from the following description and examples.

For solving the above-described problems, the inventors have constructed a massive expression system of protein having transglutaminase activity by changing the codon to that for E. coli, or preferably by using a multi-copy vector (pUC19) and a strong promoter (trp promoter).

Since MTG is expressed and secreted in the prepro-form from microorganisms of actinomycetes, the MTG does not have methionine residue corresponding to the initiation codon at the N-terminal, but the protein expressed by the above-described expression method has the methionine residue at the N-terminal thereof. To solve this problem, the inventors have paid attention to the substrate specificity of methionine aminopeptidase of E. coli, and succeeded in obtaining a protein having transglutaminase activity and free from methionine at the N-terminal by expressing the protein in the form free from the aspartic acid residue which is the N-terminal amino acid of MTG. The present invention has been thus completed.

Namely, the present invention provides a protein having a transglutaminase activity, which comprises a sequence ranging from serine residue at the second position to proline residue at the 331st position in an amino acid sequence represented by SEQ ID No. 1 wherein N-terminal amino acid of the protein corresponds to serine residue at the second position of SEQ ID No. 1.

There is provided a protein which consists of an amino acid sequence of from serine residue at the second position to proline residue at the 331st position in an amino acid sequence of SEQ ID No. 1.

There is provided a DNA which codes for said proteins.

There is provided a recombinant DNA having said DNA, in particular, a recombinant DNA expressing said DNA.

There is provided a transformant obtained by the transformation with the recombinant DNA.

There is provided a process for producing a protein having a transglutaminase activity, which comprises the steps of culturing the transformant in a medium to produce the protein having a transglutaminase activity and recovering the protein.

Taking the substrate specificity of methionine aminopeptidase into consideration, the process for producing the protein having transglutaminase activity and free of initial methionine is not limited to the removal of the N-terminal aspartic acid.

Brief Explanation of the Drawings

Fig. 1 shows a construction scheme of MTG expression plasmid pTRPMTG-01.

Fig. 2 shows a construction scheme of MTG expression plasmid pTRPMTG-02.

Fig. 3 is an expansion of SDS-polyacrylamide electrophoresis showing that MTG was expressed.

Fig. 4 shows a construction scheme of MTG expression plasmid pTRPMTG-00.

Fig. 5 shows a construction scheme of plasmid pUCN216D.

Fig. 6 shows a construction scheme of MTG expression plasmid pUCTRPMTG(+)D2.

Fig. 7 shows that GAT corresponding to Aspartic acid residue is deleted.

Fig. 8 shows that N-terminal amino acid is serine.

Detailed Description of the Preferred Embodiments

The proteins having a transglutaminase activity according to the present invention comprise a sequence ranging from serine residue at the second position to proline residue at the 331st position in an amino acid sequence represented by SEQ ID No. 1 as an essential sequence but the protein may further have an amino acid or amino acids after proline residue at the 331st position. Among these, the preferred is a protein consisting of an amino acid sequence of from serine residue at the second position to proline residue at the 331st position in an amino acid sequence of SEQ ID No. 1.

In these amino acid sequences, the present invention includes amino acid sequences wherein an amino acid or some amino acids are deleted, substituted or inserted as far as such amino acid sequences have a transglutaminase activity.

The DNA of the present invention encodes the above-mentioned proteins. Among these, the preferred is a DNA wherein a base sequence encoding for Arg at the fourth position from the N-terminal amino acid is CGT or CGC, and a base sequence encoding for Val at the fifth position from the N-terminal amino acid is GTT or GTA. Furthermore, the preferred is a DNA wherein a base sequence encoding for the N-terminal amino acid to fifth amino acid, Ser-Asp-Asp-Arg-Val, has the following sequence.

Ser : TCT or TCC

Asp : GAC or GAT

Asp : GAC or GAT

Arg : CGT or CGC

Val : GTT or GTA

In this case, the preferred is a DNA wherein a base sequence encoding for amino acid sequence of from the N-terminal amino acid to fifth amino acid, Ser-Asp-Asp-Arg-Val, has the sequence TCT-GAC-GAT-CGT-GTT.

Furthermore, the preferred is a DNA wherein a base sequence encoding for amino acid sequence of from sixth amino acid to ninth amino acid from the N-terminal amino acid, Thr-Pro-Pro-Ala, has the following sequence.

Thr : ACT or ACC

Pro : CCA or CCG

Pro : CCA or CCG

Ala : GCT or GCA

Furthermore, the preferred is a DNA comprising a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2. In this case, more preferred is a DNA consisting of a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2.

In the DNA sequences mentioned above, nucleic acids encoding an amino acid or some amino acids may be deleted, substituted or inserted as far as such DNA encodes an amino acid sequence having a transglutaminase activity.

The recombinant DNA of the present invention has one of DNA mentioned above. In this case, the preferred is a DNA having a promoter selected from the group consisting of trp, tac, lac, trc, λ PL and T7.

The transformants of the present invention are obtained by the transformation with the above-mentioned recombinant DNA. Among these, it is preferable that a transformation be conducted by use of a multi-copy vector, and that the transformants belong to Escherichia coli.

The process for producing a protein having a transglutaminase activity according to the present invention comprises the steps of culturing one of the above-mentioned transformants in a medium to produce the protein having a transglutaminase activity and recovering the protein.

The detailed description will be further made on the present invention.

(1) It is known that the expression of MTG in the cells of a microorganism is fatal. It is also known that in the high expression of the protein in a microorganism such as E. coli, the expressed protein is inclined to be in the form of inert insoluble protein inclusion bodies. Under these circumstances, the inventors made investigations for the purpose of obtaining a high expression of MTG as an inert, insoluble protein in E. coli.

A structural gene of MTG used for achieving the high expression is a DNA containing a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2. Taking the degeneration of the genetic codon, the third letter in the degenerate codon in a domain which codes for the N-

terminal portion is converted to a codon rich in adenine and uracil and the remaining portion is comprised of a codon frequently used for E. coli in order to inhibit the formation of high-order structure of mRNA, though a DNA which codes for proteins having the same amino acid sequence can have various base sequences.

A strong promoter usually used for the production of foreign proteins is used for the expression of MTG structural gene, and a terminator is inserted into the downstream of MTG structural gene. For example, the promoters are trp, tac, lac, trc, λ PL and T7, and the terminators are trpA, lpp and T4.

For the efficient translation, the variety and number in the SD sequence, and the base composition, sequence and length in the domain between the SD sequence and initiation codon were optimized for the expression of MTG.

The domain ranging from the promoter to the terminator necessitated for the expression of MTG can be produced by a well-known chemical synthesis method. An example of the base sequence is shown in SEQ ID No. 3. In the amino acid sequence of sequence No. 3, aspartic acid residue follows the initiation codon. However, this aspartic acid residue is preferably removed as will be described below.

The present invention also provides a recombinant DNA usable for the expression of MTG.

The recombinant DNA can be produced by inserting a DNA containing the structural gene of the above-described MTG in a known expression vector selected depending on a desired expression system. The expression vector used herein is preferably a multi-copy vector.

Known expression vectors usable for the production of the recombinant DNA of the present invention include pUC19 and pHS299. An example of the recombinant DNA of the present invention obtained by integrating DNA of the present invention into pUC19 is pUCTRPMTG-02(+).

The present invention also relates to various transformants obtained by the introduction of the recombinant DNA.

The cells capable of forming the transformant include *E. coli* and the like.

An example of *E. coli* is the strain JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ (lac-proAB)/F' [traD36, proAB+, lacIq, lacZ Δ M15]).

A protein having a transglutaminase activity is produced by culturing the transformant such as that obtained by transforming *E. coli* JM109 with pUCTRPMTG-02(+) which is a vector of the present invention.

Examples of the medium used for the production include 2xYT medium used in the Example given below and medium usually used for culturing *E. coli* such as LB medium and M9-Casamino acid medium.

The culture conditions and production-inducing conditions are suitably selected depending on the kinds of the vector, promoter, host and the like. For example, for the production of a recombinant product with trp promoter, a chemical such as 3- β -indoleacrylic acid may be used for efficiently working the promoter. If necessary, glucose, Casamino acid or the like can be added in the course of the culture. Further, a chemical (ampicillin) resistant to genes which are resistant to chemicals kept in plasmid can also be added in order to selectively proliferate a recombinant *E. coli*.

The protein having a transglutaminase activity, which is produced by the above-described process, is extracted from the cultured strain as follows: After the completion of the culture, the cells are collected and suspended in a buffer solution. After the treatment with lysozyme, freezing/melting, ultrasonic disintegration, etc., the thus-obtained suspension of the disintegrated cells is centrifuged to divide it into a supernatant liquid and precipitates.

The protein having a transglutaminase activity is obtained in the form of a protein inclusion body and contained in the precipitates. This protein is solubilized with a denaturant or the like, the denaturant is removed and the protein is separated and purified. Examples of the denaturants usable for solubilizing the protein inclusion body produced as described above include urea (such as 8M) and guanidine hydrochloride (such as 6 M). After removing the denaturant by the dialysis or the like, the protein having a transglutaminase activity is regenerated. Solutions used for the dialysis are a phosphoric acid buffer solution, tris hydrochloride buffer solution, etc. The denaturant can be removed not only by the dialysis but also dilution, ultrafiltration or the like. The regeneration of the activity is expectable by any of these techniques.

After the regeneration of the activity, the active protein can be separated and purified by a suitable combination of well-known separation and precipitation methods such as salting out, dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide electrophoresis, ion exchange chromatography, affinity chromatography, reversed-phase high-performance liquid chromatography and isoelectric point

electrophoresis.

(2) The present invention provides a protein having a transglutaminase activity, which has a sequence ranging from serine residue at the second position to proline residue at the 331st position in the amino acid sequence represented in SEQ ID No. 1.

The N-terminals of MTG produced by the product transformed with recombinant DNA having a DNA represented in SEQ ID No. 3 was analyzed to find that most of them contained (formyl)methionine residue of the initiation codon.

However, when a gene which encodes for an exogenous protein is expressed in E. coli, the gene is designed so that the intended protein is positioned after the methionine residue encoded by ATG which is the translation initiation signal for the gene. It is already known that N-terminal methionine residues of a natural protein obtained by the translation from genes are more efficiently cut by methionine aminopeptidase. However, the N-terminal methionine residues are not always cut in the exogenous protein.

It is known that the substrate specificity of methionine aminopeptidase varies depending on the variety of the amino acid residue positioned next to the methionine residue. When the amino acid residue positioned next to the methionine residue is alanine residue, glycine residue, serine residue or the like, the methionine residue is easily cleaved, and when the former is aspartic acid, asparagine, lysine, arginine, leucine or the like, the latter is difficultly cleaved [Nature 326, 315(1987)].

The N-terminal amino acid residue of MTG is aspartic acid

residue. When a methionine residue derived from the initiation codon is positioned directly before the aspartic acid residue, methionine aminopeptidase difficultly acts on the obtained sequence, and the N-terminal methionine residue is usually not removed but remains. However, since serine residue is arranged next to N-terminal aspartic acid in MTG, the sequence can be so designed that the amino acid residue positioned next to methionine residue derived from the initiation codon will be serine residue (an amino acid residue on which methionine aminopeptidase easily acts) by deleting aspartic acid residue. Thus, a protein having a high transglutaminase activity, from which the N-terminal methionine residue has been cleaved, can be efficiently produced.

The recombinant protein thus obtained is shorter than natural MTG by one amino acid residue, but the function of this protein is the same as that of the natural MTG. Namely, MTG activity is not lost by the lack of one amino acid. Although there is a possibility that a protein having a transglutaminase activity, from which the methionine residue has not been cleaved, gains a new antigenicity, it is generally understood that the sequence shortened by several residues does not gain a new antigenicity which natural MTG does not have. Thus, there is no problem of the safety.

In fact, a sequence of Met-Ser-Asp-Asp-Arg- was designed by deleting N-terminal aspartic acid residue from transglutaminase derived from microorganism (MTG), and this was produced in E. coli. As a result, methionine residue was efficiently removed and thereby there was obtained a protein having a sequence of Ser-Asp-

Asp-Arg-. It was confirmed that the specific activity of the thus-obtained protein is not different from that of natural MTG.

A process for producing a protein having a transglutaminase activity, which has a sequence ranging from serine residue at the second position to proline residue at the 331st position in the amino acid sequence represented in SEQ ID No. 1 will be described below.

That is, a DNA which encodes for a protein having a transglutaminase activity and having a sequence ranging from serine residue at the second position to proline residue at the 331st position in the amino acid sequence represented in SEQ ID No. 1 is employed as the MTG structural gene present on recombinant DNA usable for the expression of MTG. Concretely, a DNA having a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2 is employed.

The N-terminal sequence can be altered by an ordinary DNA recombination technique, or specific site directional mutagenesis technique, a technique wherein PCR is used for the whole or partial length of MTG gene, or a technique wherein the part of the sequence to be altered is exchanged with a synthetic DNA fragment by a restriction enzyme treatment.

The transformant thus transformed with the recombinant DNA is cultured in a medium to produce a protein having a transglutaminase activity, and the protein is recovered. The methods for the preparation of the transformant and for the production of the protein are the same as those described above.

Since the protein thus produced has a sequence of Met-Ser-. . .

from which the methionine residue is easily cleaved with methionine aminopeptidase, the methionine residue is cleaved in the cell of E. coli to obtain a protein that starts with serine residue.

Although MTG having N-terminal methionine residue is not present in the nature, the inventors have found that in some of natural MTG, aspartic acid residue is deleted to have N-terminal serine. Although a protein having N-terminal methionine residue is thus different from natural MTG in the sequence, a protein having N-terminal serine residue is included in the sequences of natural MTG and, in addition, a protein having such a sequence is actually present in the nature. Thus, it can be said that such MTG is equal to natural MTG. Namely, in the production of an enzyme to be used for foods, such as MTG, in which protein antigenicity is a serious problem, it is important to produce a protein having transglutaminase activity and also having a sequence equal to that of natural MTG, or in other words, to produce a sequence from which the N-terminal methionine residue was cleaved.

The following Examples will further illustrate the present invention, which by no means limit the invention.

Example

Mass production of MTG in E. coli:

<1> Construction of MTG expression plasmid PTRPMTG-01:

MTG gene has been already completely synthesized, taking the frequency of using codons of E. coli and yeast into consideration (J. P. KOKAI No. Hei 5-199883). However, the gene sequence thereof was not optimum for the expression in E. coli. Namely, all of codons of thirty arginine residues were AGA (minor codons). Under these conditions,

about 200 bases from the N-terminal of MTG gene were resynthesized to become a sequence optimum for the expression of *E. coli*.

As a promoter for transcribing MTG gene, *trp* promoter capable of easily deriving the transcription in a medium lacking tryptophane was used. Plasmid pTTG2-22 (J. P. KOKAI No. Hei 6-225775) for the high expression of transglutaminase (TG) gene of *Pagrus major* was obtained with *trp* promoter. The sequence in the upstream of the TG gene of *Pagrus major* was designed so that a foreign protein is highly expressed in *E. coli*.

In the construction of pTRPMTG-01, the DNA fragment from *Cla*I site in the downstream of *trp* promoter to *Bgl*II site in the downstream of *Pagrus major*'s TG expression plasmid pTTG2-22 (J. P. KOKAI Hei 6-225775) was replaced with the *Cla*I/*Hpa*I fragment of the synthetic DNA gene and the *Hpa*I/*Bam*HI fragment (small) of pGEM15BTG (J. P. KOKAI Hei 6-30771).

The *Cla*I/*Hpa*I fragment of the Synthetic DNA gene has a base sequence from *Cla*I site in the downstream of *trp* promoter of pTTG2-22 to translation initiation codon, and 216 bases from the N-terminal of MTG gene. The base sequence in MTG structural gene was determined with reference to the frequency of using codon in *E. coli* so as to be optimum for the expression in *E. coli*. However, for avoiding the high-order structure of mRNA, the third letter of the degenerated codon in the domain of encoding the N-terminal part was converted to a codon rich in adenine and uracil so as to avoid the arrangement of the same bases as far as possible.

The *Cla*I/*Hpa*I fragment of the Synthetic DNA gene was so designed

that it had EcoRI and HindIII sites at the terminal. The designed gene was divided into blocks each comprising about 40 to 50 bases so that the + chain and the - chain overlapped each other. Twelve DNA fragments corresponding to each sequence were synthesized (SEQ ID Nos. 4 to 15). 5' terminal of the synthetic DNA was phosphatized. Synthetic DNA fragments to be paired therewith were annealed, and they were connected with each other. After the acrylamide gel electrophoresis, the DNA fragments of an intended size was taken out and integrated in EcoRI/HindIII sites of pUC19. The sequence was confirmed and the correct one was named pUCN216. From the pUCN216, a ClaI/HpaI fragment (small) was taken out and used for the construction of pTRPMTG-01.

<2> Construction of MTG expression plasmid pTRPMTG-02:

Since E. coli JM109 keeping pTRPMTG-01 did not highly express MTG, parts (777 bases) other than the N-terminal altered parts of MTG gene were altered suitably for E. coli. Since it is difficult to synthesize 777 bases at the same time, the sequence was determined, taking the frequency of using codons in E. coli into consideration, and then four blocks (B1, 2, 3 and 4) therefor, each comprising about 200 bases, were synthesized. Each block was designed so that it had EcoRI/HindIII sites at the terminal. The designed gene was divided into blocks of about 40 to 50 bases so that the + chain and the - chain overlapped each other. Ten DNA fragments of the same sequence were synthesized for each block, and thus 40 blocks were synthesized in total (SEQ ID Nos. 16 to 55). 5' terminal of the synthetic DNA was phosphatized. Synthetic DNA fragments to be paired therewith were annealed, and they were connected with each other. After the

acrylamide gel electrophoresis, DNA of an intended size was taken out and integrated in EcoRI/HindIII sites of pUC19. The base sequence of each of them was confirmed and the correct ones were named pUCB1, B2, B3 and B4. As shown in Fig. 2, B1 was connected with B2, and B3 was connected with B4. By replacing a corresponding part of pTRPMTG-01 therewith, pTRPMTG-02 was constructed. The sequence of the high expression MTG gene present on pTRPMTG-02 is shown in SEQ ID No. 3.

<3> Construction of MTG expression plasmid pUCTRPMTG-02(+), (-):

Since E. coli JM109 which keeps the pTRPMTG-02 also did not highly express MTG, the plasmid was multi-copied. EcoO109I fragment (small) containing trp promoter of pTRPMTG-02 was smoothened and then integrated into HincII site of pUC19 which is a multi-copy plasmid. pUCTRPMTG-02(+) in which lacZ promoter and trp promoter were in the same direction, and pUCTRPMTG-02(-) in which they were in the opposite direction to each other were constructed.

<4> Expression of MTG:

E. coli JM109 transformed with pUCTRPMTG-02(+) and pUC19 was cultured by shaking in 3 ml of 2xYT medium containing $150 \mu\text{g/ml}$ of ampicillin at 37°C for ten hours (pre-culture). 0.5 ml of the culture suspension was added to 50 ml of 2xYT medium containing $150 \mu\text{g/ml}$ of ampicillin, and the shaking culture was conducted at 37°C for 20 hours.

The cells were collected from the culture suspension and broken by ultrasonic disintegration. The results of SDS-polyacrylamide electrophoresis of the whole fraction, and supernatant and precipitation fractions both obtained by the centrifugation are shown in Fig. 3. The high expression of the protein having a molecular weight

equal to that of MTG was recognized in the whole fraction of broken
pUCTRPMTG-02(+)/JM109 cells and the precipitate fraction obtained by
the centrifugation. It was confirmed by the western blotting that the
protein was reactive with mice anti-MTG antibody. The expression of
the protein was 500 to 600 mg/L. A sufficient, high expression was
obtained even when 3- β -indole acrylic acid was not added to the
production medium.

Further, the western blotting was conducted with MTG antibody
against mouse to find that MTG was expressed only slightly in the
supernatant fraction obtained by the centrifugation and that the
expressed MTG was substantially all in the form of insoluble protein
inclusion bodies.

<5> Construction of MTG expression plasmid pTRPMTG-00:

To prove that the change in codon of MTG gene caused a
remarkable increase in the expression, pTRPMTG-00 corresponding to
pTRPMTG-02 but in which MTG gene was changed to a gene sequence
completely synthesized before (J. P. KOKAI No. Hei 6-30771) was
constructed.

pTRPMTG-00 was constructed by connecting PvuII/PstI fragment
(small) from Pagrus major's TG expression plasmid pTRPMTG-02 with
PstI/HindIII fragment (small, including PvuII site) and PvuII/HindIII
fragment (small) of pGEM15BTG (J. P. KOKAI No. Hei 6-30771).

<6> Construction of MTG expression plasmid pUCTRPMTG-00(+), (-):

pTRPMTG-00 was multi-copied. EcoO109I fragment (small)
containing trp promoter and trpA terminator of pTRPMTG-00 was smoothened
and then integrated into HincII site of pUC19 which is a multi-copy

plasmid. pUCTRPMTG-00(+) in which lacZ promoter and trp promoter were in the same direction, and pUCTRPMTG-00(-) in which they were in the opposite direction to each other were constructed.

<7> Comparison of MTG expressions:

5 E. coli JM109 transformed with pUCTRPMTG-02 (+) or (-), pUCTRPMTG-00 (+) or (-), pTRPMTG-02, pTRPMTG-01, pTRPMTG-00 or pUC19 was cultured by shaking in 3 ml of 2xYT medium containing 150 μ g/ml of ampicillin at 37°C for ten hours (pre-culture). 0.5 ml of the culture suspension was added to 50 ml of 2xYT medium containing 150 μ g/ml of ampicillin, and the shaking culture was conducted at 37 °C for 20 hours.

10 The cells were collected from the culture suspension, and MTG expression thereof was determined to obtain the results shown in Table 1. It was found that the newly constructed E. coli containing pTRPMTG-00, pUCTRPMTG-00 (+) or (-) did not highly express MTG. This result indicate that it is necessary for the high expression of MTG to change the codon of MTG gene into a codon for E. coli and also to multi-copy the plasmid.

Table 1

Strain	MTG expression
pUCTRPMTG-02(+)/JM109	+++
pUCTRPMTG-02(-)/JM109	+++
pUCTRPMTG-00(+)/JM109	+
pUCTRPMTG-00(-)/JM109	+
pTRPMTG-02/JM109	+
pTRPMTG-01/JM109	+
pTRPMTG-00/JM109	-
pUC19/JM109	-

+++ : at least 300 mg/l

+ : 5 mg/l or below

- : no expression

<8> Analysis of N-terminal amino acid of expressed MTG:

The N-terminal amino acid residue of the protein inclusion bodies of expressed MTG was analyzed to find that about 60 % of the sequence of N-terminal was methionine residue and about 40 % thereof was formylmethionine residue. (Formyl)methionine residue corresponding to the initiation codon was removed by a technical idea described below.

<9> Deletion of N-terminal aspartic acid residue of MTG:

A base sequence corresponding to aspartic acid residue (the N-terminal of MTG) was deleted by PCR using pUCN216 containing 216 bases as the template. pUCN216 is a plasmid obtained by cloning about 216

bp's containing ClaI-HpaI fragment of N-terminal of MTG in EcoRI/HindIII site of pUC19. pF01 (SEQ ID No. 56) and pR01 (SEQ ID No. 57) are primers each having a sequence in the vector. pDELD (SEQ ID No. 58) is that obtained by deleting a base sequence corresponding to Asp residue. pHD01 (SEQ ID No. 59) is that obtained by replacing C with G not to include HindIII site. pF01 and pDELD are sense primers and pR01 and pHD01 are antisense primers.

35 cycles of PCR of a combination of pF01 and pHD01, and a combination of pELD and pR01 for pUCN216 was conducted at 94 °C for 30 seconds, at 55°C for one minute and at 72 °C for two minutes. Each PCR product was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 100 μ l of H₂O.

1 μ l of each of the PCR products was taken, and they were mixed together. After the heat denaturation at 94 °C for 10 minutes, 35 cycles of PCR of a combination of pF01 and pHD01 was conducted at 94°C for 30 seconds, at 55°C for one minute and at 72 °C for two minutes.

The second PCR product was extracted with phenol/chloroform, precipitated with ethanol, and treated with HindIII and EcoRI. After pUC19 subcloning, pUCN216D was obtained (Fig. 5). The sequence of the obtained pUCN216D was confirmed to be the intended one.

<10> Construction of the plasmid encoding for MTG which lacks N-terminal aspartic acid:

Eco0109I/HpaI fragment (small) of pUCN216D was combined with Eco0109I/HpaI fragment (large) of pUCB1-1 (plasmid obtained by cloning HpaII/BglIII fragment of MTG gene in EcoRI/HindIII site of pUC19) to obtain pUCNB1-2D. Further, ClaI/BglIII fragment (small) of pUCNB1-2D was

combined with ClaI/B/BglIII fragment (large) of pUCTRPMTG-02(+) which is a plasmid of high MTG expression to obtain pUC TRPMTG(+)D2, the expression plasmid of MTG which lacks N-termianl aspertic acid(Fig. 6). As a result, a plasmid containing MTG gene lacking GAI corresponding to aspartic acid residue as shown in Fig. 7 was obtained.

<11> Expression of the plasmid encoding for MTG which lacks N-terminal aspartic acid:

E. coli JM109 transformed with pUCTRPMTG(+)D2 was cultured by shaking in 3 ml of 2xYT medium containing 150 μ g/ml of ampicillin at 37 °C for ten hours (pre-culture). 0.5 ml of the culture suspension was added to 50 ml of 2xYT medium containing 150 μ g/ml of ampicillin, and the shaking culture was conducted at 37 °C for 20 hours. The cells were broken by the ultrasonic disintegration. The results of the dyeing with Coomassie Brilliant Blue dyeing and Western blotting with mouse antiMTG antibody of the thus obtained supernatant liquid and precipitate indicated that MTG protein lacking N-terminal aspartic acid residue was detected in the precipitate obtained by the ultrasonic disintegration, namely in the insoluble fraction. This fact suggests that MTG protein lacking N-terminal aspartic acid residue was accumulated as protein inclusion bodies in the cells.

The N-terminal amino acid sequence of the protein inclusion bodies was analyzed to find that about 90 % thereof was serine as shown in Fig.8.

The results of the analysis of N-terminal amino acids of expressed MTG obtained in <8> and <11> were compared with each other as shown in Table 2. It was found that by deleting the N-terminal

aspartic acid residue from MTG, the initiation methionine added to the N-terminal of the expressed MTG was efficiently removed.

Table 2

Strain	N-terminal amino acid			
	f-Met	Met	Asp	Ser
pUCTRPMTG-02(+)/JM109	40 %	60 %	N.D.	
pUCTRPMTG(+)/D2/JM109	N.D.	10 %	-	90 %

<12> Solubilization of MTG inclusion bodies lacking N-terminal aspartic acid residue, renaturation of activity and determination of specific activity:

MTG inclusion bodies lacking aspartic acid was partially purified by repeating the centrifugation several times, and then dissolved in 8 M urea [50 mM phosphate buffer (pH 5.5)] to obtain the 2 mg/ml solution. Precipitates were removed from the solution by the centrifugation and the solution was diluted to a concentration of 0.5 M urea with 50 mM phosphate buffer (pH 5.5). The diluted solution was further dialyzed with 50 mM phosphate buffer (pH 5.5) to remove urea. According to Mono S column test, the peak having TG activity was eluted when NaCl concentration was in the range of 100 to 150 mM. The specific activity of the fraction was determined by the hydroxamate method to find that the specific activity of the aspartic acid residue-lacking MTG was about 30 U/mg. This is equal to the specific activity of natural MTG. It is thus apparent that the lack of aspartic acid residue exerts no influence on the specific activity.